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**ASSOCIATION BETWEEN THYMIC FUNCTION AND ALLOGENEIC
HEMATOPOIETIC STEM CELL TRANSPLANTATION OUTCOME: RESULTS
OF A PEDIATRIC STUDY.**

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sjTREC in pediatric allogeneic hematopoietic stem cell transplantation

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Abstract

Robust T cell function recovery has been shown to be crucial in determining allogeneic hematopoietic stem cell transplantation outcome and there is growing evidence that the thymus plays a central role in regulating this process. We performed a long-term analysis of the role of thymic activity recovery in a population of pediatric patients undergoing allogeneic hematopoietic stem cell transplantation by signal joint T-cell receptor excision circle quantification. In this study, characterized by a long-term follow-up (median: 72 months), we found that patients with higher levels of signal joint T-cell receptor excision circles before transplantation had a statistically significant reduced risk of death compared with patients with lower values (Relative Risk: 0.31 95% CI: 0.30-0.32 $p=0.02$) and we showed that this different outcome was mainly related to a reduction of relapse incidence (14% versus 43% $p = 0.02$). Unlike from previous reports we observed no correlation between signal joint T-cell receptor excision circle levels and lymphocyte recovery. Moreover, we confirmed that only GvHD influenced thymic activity after transplantation. In conclusion, our results suggest that there is an association between pre-transplantation thymic activity and the long-term outcome of pediatric patients undergoing hematopoietic stem cell transplantation, mainly through a reduction of relapse opportunities

Introduction

Allogeneic Hematopoietic Stem Cells Transplantation (alloHSCT) is one of the best therapeutic options available for pediatric patients affected by various malignant diseases and other non-malignant disorders involving the hematopoietic system (1). T lymphocyte function recovery is a crucial event in determining the prognosis of patients undergoing alloHSCT as its prolonged impairment may be related to the occurrence of infectious

complications and, in the malignant setting, also to the recurrence of primary disease (2, 3). T cell recovery after alloHSCT typically evolves throughout two distinct phases called thymus-independent, or early phase, and thymus-dependent, or late phase. The thymus-independent phase consists in the peripheral expansion of mature T cells transferred to the patient with the graft (4, 5). The thymus-dependent phase consists in the generation of new naïve T cells from the donor-derived hematopoietic progenitors occurring in the recipient's thymus. The thymus-dependent phase accounts for the most durable reconstitution of the T-cell compartment, generates T Cell Receptor (TCR) repertoire diversity (6) and requires a functionally active thymus (7). Thymic function can be evaluated through the evaluation of the signal joint T-cell receptor excision circles (sjTRECs) by quantitative polymerase chain reaction (PCR). sjTRECs are episomal DNA fragments resulting from the deletion of the TCR δ region during TCR α locus rearrangement. As they cannot replicate and are not duplicated, they are diluted out during cell division allowing a direct evaluation of recent thymic output (8, 9). Some previous studies explored the relationship between sjTREC levels and the kinetics of the phenotypic and functional changes in peripheral T cells after alloHSCT, showing a direct correlation between sjTREC levels and the percentage of naïve T cells resulting from the thymus-dependent recovery pathway in both adults (10, 11) and pediatric (10-12) patients. sjTREC levels have also been associated with major parameters affecting the transplantation outcome such as the incidence of acute and chronic GvHD (13, 14), opportunistic infections (7, 13) and relapse (15, 16) but all these studies focused on a single parameter, in one single setting at a single time point (17) and in mixed (pediatric and adult) populations. In our study we conducted a long-term comprehensive analysis of the impact of sjTRECs on main transplantation outcome variables in a homogenous pediatric population undergoing alloHSCT.

78

79 **Materials and methods**

80 **Patients**

81 The study population included 57 patients (38 males and 19 females) aged from 0 to 22
82 years (median age: 9 years) who underwent alloHSCT between April 2006 and October
83 2008 at our Center. In order to exclude possible bias related to a too short observation
84 period, analyses were performed when the majority of patients reached a median follow up
85 of over 5 years. The Institutional Committee on Medical Ethics approved this study and
86 patients or their legal representatives provided informed consent.

87 Patients' characteristics, conditioning regimens, hematopoietic stem cell sources, donor
88 characteristics and GvHD prophylaxes are summarized in Table 1.

89 Donor selection and HLA typing were performed according to the Italian Bone Marrow
90 Donor Registry (IBMDR) Standard of Practice. In the analyses, total nucleated cells (TNC)
91 and CD34⁺ cells values were expressed in percentiles and in quartiles according to their
92 non-Gaussian distribution. Pre-transplantation co-morbidities were scored according to a
93 previously reported classification for pediatric patients (18). The patients underwent
94 clinical and hematological post-transplantation assessments according to our Center's
95 policy. Complete blood counts were performed daily until hematological recovery, twice a
96 week until day + 100 and according to the patients' clinical conditions thereafter.

97 aGvHD and cGvHD were diagnosed and classified according to previously reported
98 criteria (19, 20). To monitor patients for viral complications, cytomegalovirus (CMV),
99 Epstein-Barr virus (EBV) and adenovirus PCR were performed weekly on peripheral
100 blood.

101

sjTREC Frequency Evaluation

The day before starting the conditioning regimen, on days 90 ± 7 , 180 ± 7 and 365 ± 7 patients were evaluated for sjTREC frequency according to previously reported method (21, 22) on peripheral blood mononuclear cells (PBMC) by real time quantitative PCR (TaqMan Technology). The primer TREC sequences and probes used were: forward: 5'-TGGTTTTTGTGCCAC-3'; reverse: 5'-GTGCCAGCTGCAGGGTTT-3'; probe: 5'(FAM) CATAGGCACCTGCACCCCGTGC (TAMRA) P-3'. PCR conditions were: 2 minutes at 50°C, 10 minutes at 95°C followed by 45 cycles of amplification (95°C for 15 seconds, 60°C for 1 minute). In order to obtain absolute sjTREC quantification we prepared a standard curve by using five different concentrations of a PCR2-1TA plasmid encoding the sjTREC sequence. PCR was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystem, Foster City, CA) and data obtained were analyzed using SDS.2 software (Applied Biosystems, Foster City, CA). sjTREC values are expressed as copy number/100 ng DNA from PBMC. As the non-Gaussian distribution of sjTREC values and almost all patients enrolled in this study had median sjTREC values under the median value of age-matched controls at all the time points, all analyses were performed considering sjTREC percentiles and quartiles of the study population.

Definitions and Outcome Endpoints

The primary endpoint of this study is the assessment of the impact of sjTREC levels on the overall survival rates in a population of pediatric patients undergoing HSCT.

The secondary endpoints of the study are the assessment of sjTREC levels on both Transplant Related Mortality and Relapse Incidence and the identification of transplant-related factors able to influence sjTREC levels. Overall survival (OS) is defined as the probability of survival irrespective of the disease state at any point in time. If, at the end of the study time, the patient is still alive data are censored at the last follow-up date. Transplant Related Mortality (TRM) is defined as the probability of dying without a previous relapse occurrence. If the patient either experienced relapse or is still alive at the end of the study time, data are censored at the relapse date or at last follow-up date respectively. For malignant diseases, relapse incidence (RI) is defined as the probability of having had a relapse. If the patient either died without experiencing relapse or is still alive at the end of the study time, data are censored at the date of death or at the last follow-up date respectively. For malignancies, patients not in a first complete remission at the time of transplant and patients who had previously failed at least one first-line treatment were considered as being in an advanced disease phase, while all other patients were considered as being in an early disease phase.

Chimerism and immune recovery evaluation

Donor chimerism was determined at $+30\pm 7$ and $+60\pm 7$ days after alloHSCT on whole bone marrow mononuclear cells and at $+180\pm 7$ and $+365\pm 7$ days on PBMC by quantitative PCR of informative short tandem repeats (STR) in the recipient and donor, according to a previously described method (23). Absolute Lymphocyte numbers were obtained from complete blood count analyses and compared to normal values according to the patient's age (24). Lymphocyte recovery was defined as the first of three consecutive days with an absolute lymphocyte count over the 5th percentile of normal values for the patient's age. In

a subset of patients, we also investigated specific lymphocyte sub-population recovery at +180 days and +365 days by flow-cytometry. Helper T cell (CD3⁺CD4⁺), cytotoxic T cell (CD3⁺CD8⁺), NK cell (CD16⁺CD56⁺) and B cell (CD19⁺CD20⁺) recovery was defined as the presence of an absolute number of cells over the 5th percentile of normal values according to the patient's age (24).

Statistical analysis

OS was calculated according to the Kaplan-Meier method and the significance between the observed differences were established by the log-rank test (25).

The multivariate analysis on OS was performed using Cox's method.

TRM and Relapse rate were calculated as a cumulative incidence (CI) to adjust the analysis for competing risks: relapse and transplant-related death were considered competing risks, respectively. The differences in terms of CI were compared using Grey's test. To assess the influence of different transplant-related variables on sjTREC levels, a two-tailed Fisher Test was performed. A p-value less than 0.05 was considered statically significant. To perform multivariate analyses we selected variables reaching p-values less than 0.1 in the univariate analyses. All the statistical analyses were performed using SPSS (IBM Corp. 2012, Armonk, NY, USA), NCSS (Hintze, 2001; NCSS PASS, Number Cruncher Statistical System, Kaysville, UT, USA) and R 2.5.0 software packages.

Results

sjTREC Frequency

Median sjTREC values were 16 (0-1684), 1 (0-160), 14 (0-553) and 201 (0-1006) sjTREC copies/100 ng DNA before HSCT and at day +90, day +180 and day +365, respectively.

In order to identify transplant-related factors associated to the frequency of sjTRECs, we evaluated the impact of different variables on median sjTREC values before HSCT and then at different time points (Table 2).

Overall Survival

As at March 2014, the median follow-up time of patients who are still alive is 72 months (42-90). The overall Survival (OS) rate at 7 years of the entire study population is 70% (95%CI: 58-82). We found a statistically significant relationship between sjTREC frequency before transplantation and 7 years OS. Patients with sjTRECs below the 50th percentile of the study population values before HSCT had an OS of 56% (95%CI: 38-73), while patients with sjTRECs above the 50th percentile had an OS of 85% (95%CI: 71-98) (p=0.02) (Table 3 and Figure 1). Moreover, before transplantation, it was possible to perform a more extended analysis considering the sjTREC frequency sub-grouped into quartiles: patients with sjTREC values in the 1st, 2nd, 3rd and 4th quartiles had OS rates of 40% (95%CI: 14-65), 71% (95% CI: 47-94), 87% (95%CI: 69-100) and 83% (95%CI: 63-100) respectively, and these differences were statistically significant (p= 0.009). Considering OS at 2 years, we found that there is a difference according to pre HSCT sjTREC levels: patients with sjTREC levels under the median value of the study population had an OS of 73% (95% CI: 57-89), while patients with sjTREC levels over the median value of the study population had an OS of 89 % (95% CI: 57-89), although at this time point this difference is not statistically significant (p= 0.13). Restricting 2-years OS analysis according to pre HSCT sjTREC levels only to the cohort of patients affected by malignant diseases, we also highlighted a difference (p= 0,14) but not statistically significant. Female patients showed better OS compared to male patients [89% (CI95%:

75-100) versus 60% (CI95%: 44-76) $p=0.035$] and patients in an early disease phase had better OS compared to patients in advanced disease phases [100% versus 60% (95%CI: 44-76) $p= 0.04$]. All the other variables investigated in the univariate analysis (Table 3) showed no correlation with OS. In particular we did not observe a correlation between OS or sjTREC levels at +90, +180 and +365 days after HSCT (Table 3). To perform multivariate analysis we selected from among variables listed in Table 3, those reaching a p value less than 0.1 in the univariate analysis (sex, co-morbidities, disease phase at HSCT and pre HSCT sjTREC levels). In the multivariate analysis, sjTREC levels before transplantation and pre HSCT co-morbidities were the only variables we found to be associated with OS: the patients with higher sjTRECs values showed a statistically significant reduced risk of death compared with patients with lower sjTRECs values (Relative Risk: 0.49 95%CI: 0.48-0.5 $p=0.03$) and patients in low risk group according to Smith et al (18) showed a statistically significant reduced risk of death compared with patients in the intermediate risk group (Relative Risk: 2.5 95%CI: 2.49-2.5 $p=0.03$). In multivariate analysis sex and the disease phase showed no statistically significant relationship with OS.

Transplant Related Mortality

The overall Transplant Related Mortality (TRM) was 5% (95%CI: 2-16). In the univariate analysis sjTREC levels before transplantation and sjTREC levels at +90 days did not show any correlation with the TRM. At +180 days from the transplant the patients with sjTRECs values under the 50th percentile had TRM of 11% (95%CI: 4-32) versus TRM of 0 of patients with sjTREC values over the 50th percentile. Likewise, at +365 days patients with sjTREC values under the 50th percentile had TRM of 10% (95%CI: 1-37) versus TRM of

0 of patients with sjTREC values over the 50th percentile. These differences in terms of TRM were not statistically significant ($p=0.1$ and $p=0.17$ respectively) (Table 4).

Relapse Incidence

For malignant disease, the overall Relapse Incidence (RI) was 30% (95% CI: 20-46). sjTREC levels before transplantation were related to the relapse. Patients with sjTREC levels below the 50th percentile of the study population relapsed in 43% of cases (95% CI: 28-66), while 14% patients with sjTREC levels above the 50th percentile experienced a relapse (95% CI: 5-41) and this difference was statistically significant ($p=0.02$) (Table 4 and Figure 2). Considering sjTREC levels before the transplant sub-grouped in quartiles, patients with sjTREC levels in the 1st, 2nd, 3rd and 4th quartiles had a relapse in 64% (95% CI: 43-95), 21% (95% CI: 8-58), 14% (95% CI: 4-51) and 14% (95% CI: 2-88) of cases, respectively, and this difference was statistically significant ($p=0.01$). sjTREC levels at +90, +180 and +365 days were not related to the recurrence. Among other variables investigated by univariate analysis the patient's gender showed a relationship with RI: male patients relapsed in 40% (95% CI: 27-60) while female patients relapsed in 7% (95% CI: 1-47) $p=0.03$ (Table 5). To perform multivariate analysis, we selected, from among the variables listed in Table 5, those reaching a p value less than 0.1 in the univariate analysis (sex and pre-HSCT sjTREC levels) and disease phase at HSCT. In the multivariate analysis sjTREC levels before transplantation were the only variables we found to be statistically associated (RR 0.0 $p < 0.0001$) with RI.

Chimerism and Immune Recovery

All the patients showed sustained engraftment and we did not observe any cases of either

early- or late-graft loss. Patients enrolled in the study reached the 5th percentile of normal lymphocyte values for the patient's age in a median of 70 days (range: 21-420) with no differences related to pre-HSCT sjTREC levels: 73 days (range: 25-420) for patients with sjTRECs over the 50th percentile before HSCT versus 65 days (range: 21-385) for patients with sjTRECs under the 50th percentile before HSCT. Considering the lymphocyte subpopulations, the proportion of patients who reached the 5th percentile of normal values for their ages of CD3⁺CD4⁺, CD3⁺CD8⁺, CD16⁺CD56⁺ and CD19⁺CD20⁺ cells was 17%, 65%, 82% and 60% at day +180 and 70%, 85%, 88% and 77% at day +365, respectively, with no differences related to pre-HSCT sjTREC levels.

Discussion

T cell function recovery has been shown to be one of the most important factors in determining the prognosis of patients undergoing alloHSCT and the role of the thymus in this process is well established. Previous studies focused on Severe Combined Immunodeficiency Disease Screening Programs in newborns (26) and on the management of patients affected by HIV and undergoing Highly Active Antiretroviral Therapy (HAART) (27) indicate that sjTREC quantification is an easy, sensible and reliable technique to evaluate immunological function and also to drive therapeutic interventions in these settings. Although the experience of alloHSCT is more limited, there is growing evidence that sjTREC quantification by PCR is one of the easiest and most reliable methods to evaluate thymic activity after alloHSCT as well. This is because, compared to other techniques (i.e. flow cytometry), this method offers the advantage of not being influenced by any phenomena which typically occur after transplantation, such

as the opportunity of T memory cells to revert into a T naïve phenotype in case of recurrent herpes virus infection (28), the possibility of T naïve cells to maintain their phenotype while acquiring T memory cells' function (29) and the maintenance of CD31 expression during CD4⁺ cells cytokine-driven proliferation (30). Even though other studies have already shown that there is a correlation between sjTREC levels and the various phases of immune recovery after alloHSCT (10-12) and that patients with a more efficient thymic function show a better prognosis compared to others (13, 31), there are still very few studies specifically concerning pediatric patients and considering that aging is a major parameter impacting thymic function (9, 32), childhood may be considered an ideal setting to further consolidate these data. In the present study we analyzed in a population of pediatric patients undergoing alloHSCT, the role of sjTREC levels on the OS and found that patients with more efficient thymic function before the transplantation had better long term OS compared to others. However, sjTREC levels after transplantation, according to our data, did not have any influence on OS at any of the time-points considered. To our knowledge there are only two previous studies that specifically investigate the impact of sjTRECs on OS. Clave *et al* demonstrated a correlation between pre-transplantation sjTREC levels and OS but even though a high number of cases were reported (n=102), only sibling recipients were included, the patients' median age was higher and only pre-transplantation sjTREC levels (17) were considered. Olkinuora *et al* in 66 pediatric patients who underwent alloHSCT reported a shorter median survival time for patients with low sjTREC levels at different time points (both before and after alloHSCT) compared to patients with high sjTREC levels, but their study lacked a real survival analysis performed with the Kaplan-Meier method and the follow-up is shorter (33). We basically confirmed a correlation between sjTREC levels

and OS in a more homogeneous and younger population also including unrelated transplant recipients and, by extending the follow-up to a median time of 72 months, we highlighted that among pediatric patients long-term survival is closely related to pre-HSCT sjTREC levels. However, as a large proportion of the patients enrolled in our study have acute lymphoblastic leukemia (ALL) that tends to relapse in the first months after transplantation, the correlation between sjTREC levels and OS seems to be less strong in the short term moreover the small number of patients affected by non malignant disorders included in the study population might introduce some potential confounding factors that are to be considered. In order to understand whether the mortality reduction we observed was attributable to a reduction of either TRM or RI, we analyzed in the same population the impact of sjTREC levels on these two outcome parameters. In line with other authors' findings, we observed a strong correlation between pre-transplantation thymic functions and RI (15, 16). However, unlike these authors, who investigated the role of sjTRECs in only one specific setting, surprisingly, we did not observe a correlation between post-transplantation thymic activity and RI. This difference might be related to the heterogeneity of our study population that included bone marrow, peripheral blood stem cells and cord blood recipients. One possible objection to our observations might be that reduced sjTREC frequency before alloHSCT might be related to more intense treatments administered because of a more aggressive disease and that OS and RI differences might only be related to a more advanced disease phase. However, via multivariate analysis we were able to show how sjTREC levels before transplantation are statistically associated to OS and RI independently from other variables, including the presence of an advanced disease phase, and, by correlation analysis, we excluded a link between the disease phase at transplantation and the time between diagnosis and HSCT and sjTREC frequency

before alloHSCT. In multivariate analysis we found that the only other variable associated with OS was the presence of co-morbidities as scored by Smith (18). Unlike previous observations (7, 13, 14, 33, 34), we did not observe a relationship between sjTRECs and TRM, probably because the very low incidence of these complications in our study population, related to the lower frequency of co-morbidities in young individuals. Finally, to clarify whether increased OS and reduced RI of patients with higher pre-HSCT sjTREC levels were related to an improved immune recovery, we evaluated the absolute lymphocyte count recovery and, surprisingly, we did not observe any differences between the patients with values over the 50th percentile or patients with values under the 50th percentile. Our data contrast with previous reports (12) but this might be due to our smaller sample size and might be related to the differences in the recovery of different lymphocyte subsets (T, B, NK) after HSCT. By analyzing transplant related factors that influence sjTREC frequency, we confirmed previously reported observations on adults (35) confirming GvHD as one of the most limiting factors in determining sjTREC levels after transplantation. However, according to our analysis, sjTREC reductions after HSCT was not statistically correlated with any worsening in terms of OS, TRM or Relapse.

The main weakness of our study is that we analyzed sjTREC frequency on whole PBMCs while other authors performed the same analysis more precisely on selected lymphocyte populations (i.e. CD3⁺, CD3⁺ CD4⁺, CD3⁺ CD8⁺).

Another limit of our study is that in the series of patients we have described, the majority of the patients was affected by ALL but no cases of T cell leukemia were included and this may have some consequences in terms of both RI and OS.

In conclusion our results confirm that thymic function does play an important role in determining the prognosis of pediatric patients undergoing alloHSCT, suggesting that an efficient thymic function before transplantation is related to improved long-term OS, mainly through a reduction of relapse opportunities. Obviously, larger and more accurate studies are needed both to confirm these observations and to identify the mechanism driving them, in order to find solutions aimed at improving T cell recovery after alloHSCT.

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Tables

Table 1. Patients' and Hematopoietic Stem Cell Transplantation details

		n	%
Sex	Male	38	67 %
	Female	19	33 %
Disease	ALL	23	40 %
	AML	8	14 %
	Inborn errors	6	10 %
	Solid Tumors	6	10 %
	Lymphoma	5	9 %
	MDS & JMML	4	7 %
	HLH	2	3,5 %
	SAA	2	3,5 %
	CML	1	1 %
Phase*	Early	8	17 %
	Advanced	39	83 %
Co-morbidity score (18)	0	44	79 %
	1-2	13	23 %
	3+	0	
Conditioning Regimen	TBI based	31	54 %
	Bu based	13	23 %
	Others	13	23 %
HSC source	BM	46	81 %
	CB	8	14 %
	PBSC	3	5 %
Donor	Sibling	21	37 %
	MUD	17	30 %
	MMUD	11	19 %
	CB	8	14 %
GvHD prophylaxis	CyA-MTX-ATG	27	48 %
	CyA	12	21 %
	CyA-MTX	8	14 %
	CyA-ATG-MMF	4	7 %
	CyA-ATG-PDN	3	5 %
	Others	3	5 %

*for malignant diseases only

ALL: Acute Lymphoblastic Leukemia, AML: Acute Myelogenous Leukemia, MDS: myelodysplasia, JMML: Juvenile Myelo Monocytic Leukemia, HLH: Hemophagocytic Lymphohistiocytosis SAA: Severe Aplastic Anemia, CML: Chronic Myelogenous Leukemia, TBI: Total Body Irradiation, Bu: busulfan, BM: bone marrow, CB: cord blood, PBSC: peripheral blood stem cells, MUD: matched unrelated donor, MMUD: mismatched unrelated donor, CyA: cyclosporine, MTX: methotrexate, ATG: antithymocyte globulins, MMF: mycophenolate mofetil, PDN: prednisone.

Table 2. sjTREC frequency

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HSCT hematopoietic stem cell transplantation, ATG: antithymocyte globulins *for malignant diseases only

		No. of patients with sjTREC level < of median value of the study population	No. of patients with sjTREC level > of the median value of the study population	p
Pre-HSCT (pts evaluable 57)	Age			
	0-5 years (n=15)	6 (40%)	9 (60%)	
	6-8 years (n=11)	3 (27%)	8 (73%)	
	9-14 years (n= 16)	8 (50%)	8 (50%)	
	>14 years (n= 15)	8 (53%)	7 (47%)	
	Disease			0.03
	Malignant (n= 47)	28 (60%)	19 (40%)	
	Non-malignant (n= 10)	2 (20%)	8 (80%)	
	Comorbidities(18)			0.21
	Low risk (n=44)	21 (48%)	23 (52%)	
	Intermediate risk (n= 13)	9 (70%)	4 (30%)	
	Disease Phase*			0.005
Day +90 (pts evaluable 57)	Early (n= 8)	1 (12%)	7 (88%)	
	Advanced (n=39)	27 (69%)	12 (31%)	
	Time from diagnosis to HSCT			0.57
	< 6 months (n=25)	16 (64%)	9 (36%)	
	> 6 months (n=22)	12 (54%)	10 (46%)	
	ATG			0.02
	Yes (n= 37)	25 (68%)	12 (32%)	
	No (n= 20)	5 (25%)	15 (75%)	
	Viral Infection			0.01
	Yes (n=30)	21 (70%)	9 (30%)	
	No (n= 27)	9 (33%)	18 (67%)	
Day + 180 (pts evaluable 57)	grade II-IV acute GvHD			0.03
	Yes (n=21)	15 (71%)	6 (29%)	
	No (n= 36)	16 (44%)	20 (56%)	
Day + 365 (pts evaluable 43)	Age			0.03
	0-5 years (n=9)	3 (33%)	6 (64%)	
	6-8 years (n=10)	3 (30%)	7 (70%)	
	9-14 years (n=14)	7 (50%)	7 (50%)	
	>14 years (n=10)	9 (90%)	1 (10%)	
	cGvHD			0.02
	Yes (n= 6)	6 (100%)	0	
	No (n= 37)	16 (43%)	21 (57%)	
	Viral Infection			0.03
	Yes (n=24)	16 (67%)	8 (33%)	
	No (n= 19)	6 (32%)	13 (68%)	

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Table 3. Overall Survival

Variable	n	Events	7 years OS	95% CI	Log Rank Test
<i>sjTREC_s pre HSCT</i>					
<50 th percentile	30	13	56 %	(38-73)	p= 0.02
>50 th percentile	27	4	85 %	(71-98)	
<i>sjTREC_s +90 days</i>					p= 0.97
<50 th percentile	30	9	70 %	(54-86)	
>50 th percentile	27	8	70 %	(52-88)	
<i>sjTREC_s +180 days</i>					p= 0.1
<50 th percentile	29	7	60 %	(42-78)	
>50 th percentile	25	8	80 %	(66-94)	
<i>sjTREC_s +365 days</i>					p= 0.6
<50 th percentile	20	4	77 %	(59-95)	
>50 th percentile	18	6	83 %	(65-100)	
<i>Sex</i>					p= 0.035
Male	38	15	60 %	(44-76)	
Female	19	2	89 %	(75-100)	
<i>Age</i>					p= 0.28
0-5 years	15	5	63 %	(36-90)	
6-8 years	11	4	64 %	(36-91)	
9-14 years	16	2	87 %	(71-100)	
> 15 years	15	6	60 %	(34-85)	
<i>Disease</i>					p= 0.14
Malignant	47	16	65 %	(53-81)	
Non-malignant	10	1	90 %	(63-100)	
<i>Co-morbidity score</i>					p< 0.0001
Low risk group	44	7	84 %	(83-84)	
Intermediate risk group	13	10	23 %	(0-46)	
<i>Disease phase*</i>					p= 0.04
Early	8	0	100 %		
Advanced	39	16	60 %	(44-76)	
<i>Time between diagnosis and HSCT*</i>					p = 0.55
< 6 months	25	7	73 %	(56-90)	
> 6 months	22	9	60 %	(40-80)	
<i>TBI</i>					p= 0.51
Yes	31	8	73 %	(57-89)	
No	26	9	65 %	(47-83)	
<i>HSC source</i>					p= 0.27
BM	46	16	65 %	(51-79)	
PBSC	3	1	87 %	(63-100)	
CB	8	0	100 %		
<i>TNC</i>					p= 0.65
<50 th percentile	29	9	69 %	(51-87)	
>50 th percentile	28	8	71 %	(53-89)	
<i>CD34⁺ cells</i>					p= 0.78
<50 th percentile	30	9	70 %	(54-86)	
>50 th percentile	27	8	69 %	(51-87)	

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All the variables potentially able to influence OS were evaluated: sjTREC levels before alloHSCT patient's sex, co-morbidities and disease phase showed a statistically significant ($p < 0.05$) correlation with OS. sjTREC_s: signal joint T cell receptor excision circles, TBI: Total Body Irradiation, HSC: hematopoietic stem cells, BM: bone marrow, PBSC: peripheral blood stem cells, CB: cord blood, TNC: total nucleated cells

496 **Table 4. Transplant-related mortality (TRM) univariate analysis**

Variable	TRM	95% CI	Grey test
<i>sjTRECs pre-HSCT</i>			p = 0.46
<50 th percentile	3 %	(0-23)	
>50 th percentile	7 %	(2-28)	
<i>sjTRECs +90 days</i>			p = 0.60
<50 th percentile	7 %	(2-25)	
>50 th percentile	4 %	(0-26)	
<i>sjTRECs +180 days</i>			p= 0.10
<50 th percentile	11 %	(4-32)	
>50 th percentile	0		
<i>sjTRECs +365 days</i>			p= 0.17
<50 th percentile	10 %	(1-37)	
>50 th percentile	0		

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499 Single joint T cells receptor excision circles (sjTRECs) level showed no statistically significant correlation (p< 0.05) with TRM at any
500 of the time points considered

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Table 5. Relapse Incidence for malignant diseases: univariate analysis

Variable	n	Events	Relapse Incidence	95% CI	Grey test
<i>sjTRECs pre-HSCT</i>					p = 0.02
<50 th percentile	28	12	43%	(28-66)	
>50 th percentile	19	3	14%	(5-41)	
<i>sjTRECs +90 days</i>					p = 0.60
<50 th percentile	26	7	26%	(14-49)	
>50 th percentile	21	8	33%	(18-61)	
<i>sjTRECs +180 days</i>					
<50 th percentile	27	10	37%	(15-52)	p = 0.34
>50 th percentile	20	5	25%	(5-46)	
<i>sjTRECs +365 days</i>					p = 0.36
<50 th percentile			11%	(3-41)	
>50 th percentile			23%	(8-62)	
<i>Sex</i>					p= 0.03
Male	34	14	41%	(27-61)	
Female	13	1	8 %	(1-50)	
<i>Age</i>					p= 0.58
0-5 years	12	4	33 %	(14-69)	
6-8 years	8	4	50%	(21-92)	
9-14 years	15	3	20 %	(7-55)	
> 15 years	12	4	33 %	(15-74)	
<i>Disease phase</i>					p= 0.20
Early	8	1	12%	(2-78)	
Advanced	39	14	35%	(22-52)	
<i>Time between diagnosis and HSCT</i>					p= 0.67
< 6 months	25	7	28%	(15-52)	
> 6 months	22	8	36%	(21-63)	
<i>HSC source</i>					p= 0.29
BM	39	14	36%	(24-55)	
CB	7	1	14%	(2-87)	
PBSC	1	0	0		
<i>Donor</i>					p= 0.24
Related	17	7	41%	(23-73)	
Unrelated	30	8	27%	(15-48)	
<i>aGvHD</i>					p= 0.41
Yes	20	5	25%	(12-53)	
No	27	15	37%	(23-60)	
<i>cGvHD</i>					p= 0.57
Yes	8	2	25%	(7-83)	
No	39	13	32%	(20-50)	

Univariate analysis of variables potentially able to influence Relapse Incidence : single joint T cell receptor excision circles (sjTREC) before the transplantation and patients' sex were statistically related to RI incidence ($p < 0.05$). HSC: hematopoietic stem cell, BM: bone marrow, PBSC: peripheral blood stem cells, CB: cord blood, aGvHD: acute graft versus host disease, cGvHD: chronic graft versus host disease

Figure legends

Figure 1. Overall Survival according to sjTREC levels

Patients with sjTRECs over the 50th percentile before HSCT (continuous line) showed a statistically significant increased survival rate compared to patients with sjTRECs under the 50th percentile (dotted line) at same time point.

Figure 2. Relapse rate according to sjTREC levels

Patients with sjTRECs over the 50th percentile before HSCT (continuous line) showed a statistically significant reduced relapse rate compared to patients with sjTRECs under the 50th percentile (dotted line) at same time point.

Figures

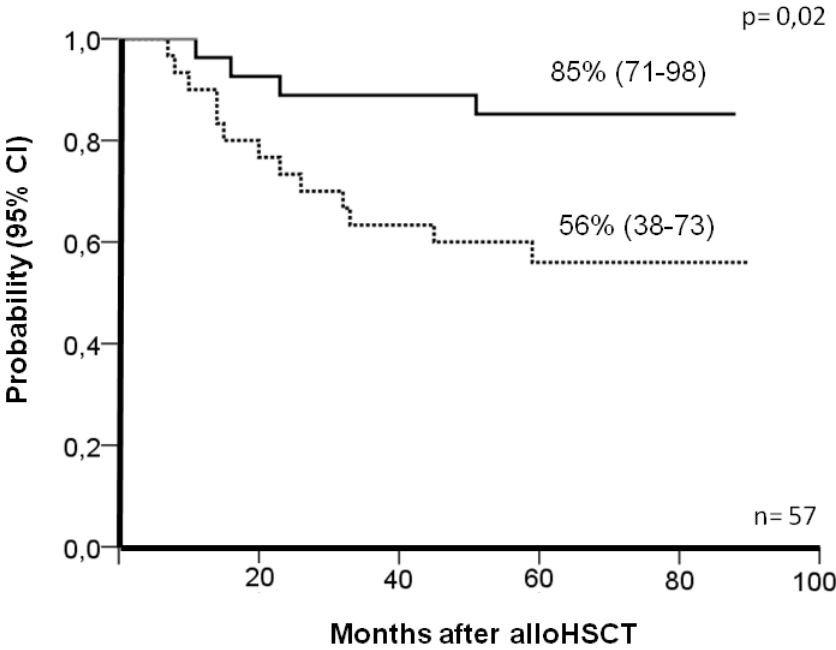


Figure 1

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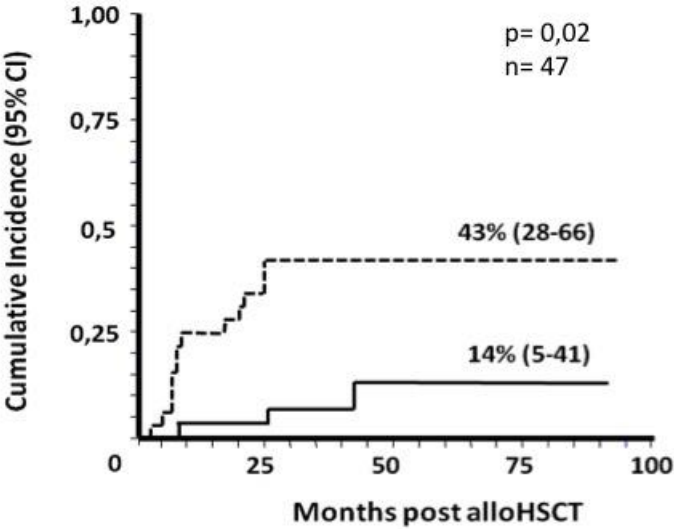


Figure 2

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